# Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial

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## Summary

**Background** Antigen sparing is regarded as crucial for pandemic vaccine development because worldwide influenza vaccine production capacity is limited. Adjuvantation is an important antigen-sparing strategy. We assessed the safety and immunogenicity of a recombinant H5N1 split-virion vaccine formulated with a proprietary adjuvant system and investigated whether it can induce cross-reactive immunity.

**Methods** Two doses of an inactivated split A/Vietnam/1194/2004 NIBRG-14 (recombinant H5N1 engineered by reverse genetics) vaccine were administered 21 days apart to eight groups of 50 volunteers aged 18–60 years. We studied four antigen doses (3·8 μg, 7·5 μg, 15 μg, and 30 μg haemagglutinin) given with or without adjuvant. Blood samples were collected to analyse humoral immune response. Adverse events were recorded up through study day 51. Safety analyses were of the whole vaccinated cohort and immunogenicity analyses per protocol. This trial is registered with the ClinicalTrials.gov, number NCT00309634.

**Findings** All eight vaccine formulations had a good safety profile. No serious adverse events were reported. The adjuvanted vaccines induced more injection-site symptoms and general symptoms than did the non-adjuvanted vaccines, but most were mild to moderate in intensity and transient in nature. The adjuvanted formulations were significantly more immunogenic than the non-adjuvanted formulations at all antigen doses. At the lowest antigenic dose (3·8 μg), immune responses for the adjuvanted vaccine against the recombinant homologous vaccine strain (A/Vietnam/1194/2004 NIBRG-14, clade 1) met or exceeded all US Food and Drug Administration and European Union licensure criteria. Furthermore, 37 of 48 (77%) participants receiving 3·8 μg of the adjuvanted vaccine seroconverted for neutralising antibodies against a strain derived by reverse genetics from a drifted H5N1 isolate (A/Indonesia/5/2005, clade 2).

**Interpretation** Adjuvantation conferred significant antigen sparing that could increase the production capacity of pandemic influenza vaccine. Moreover, the cross-clade neutralising antibody responses recorded imply that such a vaccine could be deployed for immunisation before a pandemic.

## Introduction

The spread of H5N1 avian influenza from Asia to other parts of the world is a continuing concern for global health.[1] The causative H5N1 virus (or a closely related strain) is regarded as the most probable source for the next human influenza pandemic.[2–6] This highly pathogenic virus has an H5 subtype of haemagglutinin antigen, against which most of the population has no immunity. The pandemic potential of H5N1 was shown when it first infected human beings in Hong Kong in 1997.[7,8] Up to now, 318 cases have been reported to WHO, 192 (60%) of which have been fatal.[9] Therefore, WHO considers that we are now in phase 3 (of 6) of a pandemic alert, based on the evolution of the virus into a strain that is capable of efficient human-to-human transmission.[5,10]

Influenza vaccines are expected to form the main prophylactic measure against pandemic influenza,[11] and a vast number of doses will be needed to meet the worldwide demand. Conventional seasonal influenza vaccines against circulating interpandemic strains are not expected to protect against H5N1; thus safe and effective H5N1 influenza vaccines are urgently needed. The main strategy to develop such vaccines uses reverse genetics to generate attenuated strains that express H5 surface antigens,[11–13] but the yield of vaccine antigen from these strains is typically less than half of that achieved with interpandemic strains.[14] Furthermore, avian H5 haemagglutinin seems to be an inherently poor immunogen in people, and antigen doses greater than the 15 μg present in seasonal influenza vaccines are needed to induce protective antibody concentrations.[15–18] Furthermore, because the population is largely immunologically naive to the H5 haemagglutinin, the one-dose schedule routinely used for seasonal influenza vaccines is unlikely to be sufficient. Clinical studies with split-virion or killed whole-virion vaccines based on H5N1, and other vaccines based on non-H5N1 avian strains, all show that two immunisations are necessary to elicit the amount of immunity required by licensure criteria in individuals who are immunologically naive to these strains.[15–21]

These factors further jeopardise the global manufacturing capacity to supply sufficient influenza antigen to meet the pandemic demand.[14] Clearly, new formulations that require less antigen per dose are needed. The use of adjuvant to improve immunogenicity is a crucial antigen-sparing strategy. Although a trial with a whole-virion H5N1 vaccine adjuvanted with aluminium showed promising results,[19] the use of aluminium as an adjuvant with a split-virion H5N1 vaccine suggested that doses of 30 μg were still needed to induce protective antibody concentrations in most individuals.[17]

We aimed to assess the safety and immunogenicity of a recombinant H5N1 split-virion vaccine formulated with a proprietary adjuvant system and show whether it can induce cross-reactive immunity against a strain derived by reverse genetics from a drifted clade 2 H5N1 strain.

## Methods

### Participants

We undertook this observer-blind randomised trial at the Centre for Vaccinology, Ghent University and Hospital, Ghent, Belgium. Eligible participants were clinically healthy male or female volunteers aged between 18 and 60 years at the time of first vaccination. All participants provided written informed consent.

We obtained regulatory approval for the study from the Belgian health authority (Federal Public Service, Health, Food Chain Safety and Environment). The Ethics Committee of Ghent University Hospital reviewed the protocol and other relevant study documentation.

### Procedures

The vaccine used in this study was the monovalent A/H5N1, inactivated, split-virion influenza vaccine manufactured by GlaxoSmithKline (GSK) Biologicals, Sächsisches Serumwerk (Dresden, Germany). The vaccine seed virus was an H5N1 reassortant reference virus (A/Vietnam/1194/2004 NIBRG-14) derived by reverse genetics from the highly pathogenic avian strain A/Vietnam/1194/2004 by the UK National Institute for Biological Standards and Control and recommended as suitable for use as a prototype pandemic influenza vaccine strain by the European Union Committee for Medicinal Products for Human Use (CHMP).[11]

The seed virus was propagated on fertilised hens' eggs, and the candidate vaccine was produced according to the licensed manufacturing and testing process for the interpandemic trivalent split-virion influenza vaccine Fluarix (GSK). The vaccine antigens were formulated as a 0·5 mL liquid in monodose vials at four different doses of H5 haemagglutinin, and the final formulations were prepared just before vaccination by addition of 0·5 mL diluent or 0·5 mL adjuvant. The adjuvant, manufactured by GSK Biologicals (Rixensart, Belgium), was a 10% (by volume) oil-in-water based emulsion. The oil phase contained 5% DL-α-tocopherol and squalene, and the aqueous phase contained 2% of the non-ionic detergent polysorbate 80 (Tween 80).

Since the adjuvanted and non-adjuvanted formulations differed in appearance, the blinding was maintained by all vaccinations being done by specific study personnel who did not take part in the assessment of safety or immunogenicity. Participants were randomly assigned to receive two doses of vaccine 21 days apart. Vaccines contained 3·8 μg, 7·5 μg, 15 μg, or 30 μg H5 haemagglutinin antigen with or without adjuvant. A randomisation list was generated by the sponsor by SAS program (version 8.2) and used to number the vaccines. We used a randomisation blocking scheme, with a block size of eight in an equal allocation ratio to ensure that balance between treatments was maintained and all participants were randomly allocated to groups. The randomisation algorithm used centre and age groups (18–30 years *vs* 31–60 years) as minimisation factors with equal weight in the minimisation procedure. The treatment allocation at the study site was done through a central randomisation call-in system on the internet.

The vaccines were given by intramuscular injection into the deltoid muscle of the non-dominant arm. To ensure maximum safety, vaccinations were done step-by-step by use of a staggered design. In the first stage, we randomly allocated 120 participants to the six groups (20 per group) receiving non-adjuvanted and adjuvanted vaccine formulations containing the three lowest doses of antigen. Safety and reactogenicity data were obtained for these participants for up to 7 days after the first vaccine dose and assessed before we proceeded with enrolment of people for the first dose of the 30 μg antigen formulations and of further individuals for the first dose of the 3·8 μg, 7·5 μg, and 15 μg formulations. This safety assessment procedure was repeated for the administration of the second vaccine dose.

Participants were given diary cards to record the presence and intensity of adverse events (pain, redness, swelling, induration, and ecchymosis) at the injection site and general adverse events (arthralgia, fatigue, fever, headache, muscle aches, shivering, and sweating) that they had during the first 7 days after vaccination. The diameters of any redness, swelling, induration, and ecchymosis at the injection site, and daily body temperature, were recorded. The intensities of other adverse events were recorded according to a standard three-grade scale: easily tolerated (on touch for pain at the injection site), interferes with normal activity (when limb is moved for pain at the injection site), and prevents normal activity. Data were also obtained on the occurrence and intensity of any unsolicited signs or symptoms arising within 21 days after the first vaccination and 30 days after the second vaccination. Data for serious adverse events were obtained prospectively up to study day 51. Long-term follow-up of these participants will continue until study day 180. All safety and reactogenicity observations were reviewed after interviews with individuals at the scheduled study visits.

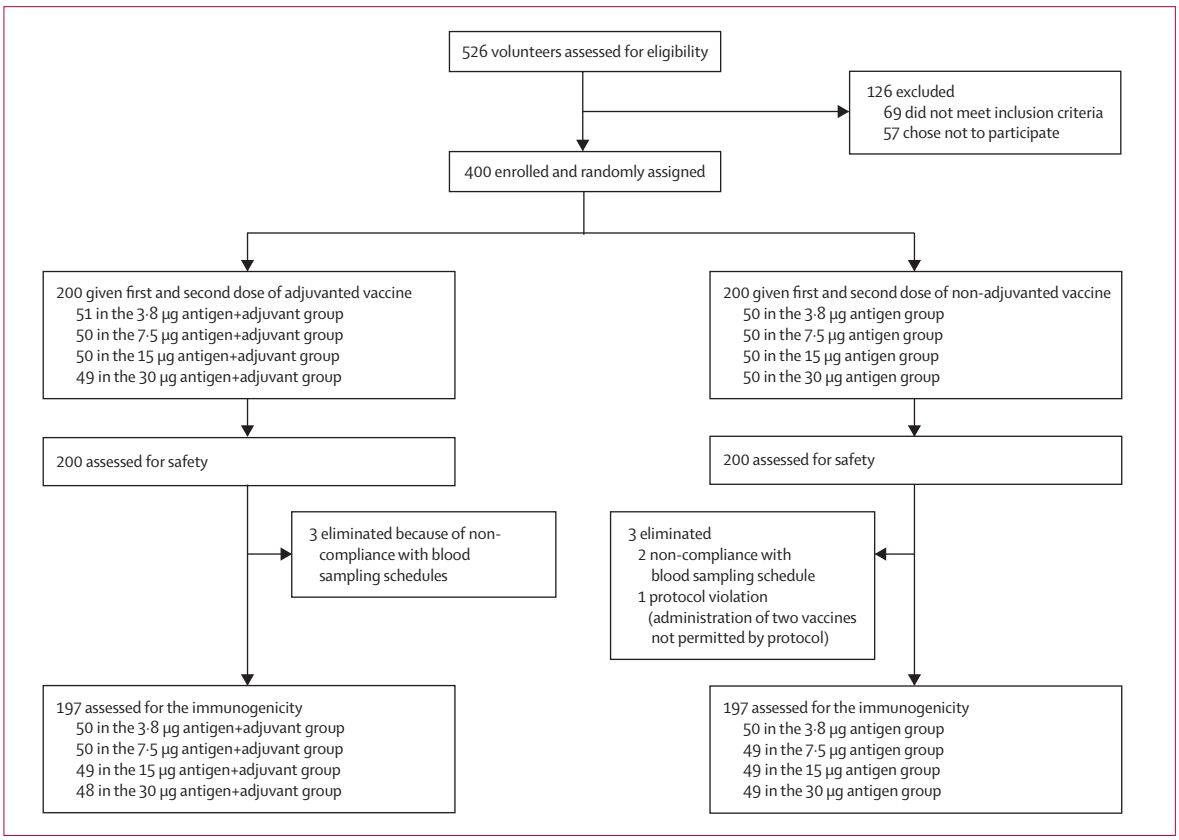


*Figure 1:* **Trial profile**

We assessed humoral immune responses by measurement of haemagglutination-inhibition antibody (HAI) titres. Serum samples were obtained for antibody titrations just before every vaccination and 21 days later. They were stored at –20°C until blinded analyses were done in the laboratory at GSK Biologicals, Sächsisches Serumwerk (Dresden, Germany). HAI assays were done according to established procedures[21–23] modified for the use of equine rather than avian erythrocytes.[24] Non-specific inhibitors of agglutination were removed by heat treatment and addition of a receptor-destroying enzyme. Serum samples were tested in two-fold dilutions starting with an initial dilution of 1 in 10. The serum titre was expressed as the reciprocal of the highest dilution that showed complete inhibition of haemagglutination. All serum samples were tested in duplicate.

Neutralisation assays were done as described previously[23] but with a haemagglutination step to detect viral replication in the supernatants.[21] Serum samples were tested in two-fold dilutions starting with an initial dilution of 1 in 28. The serum titre was expressed as the reciprocal of the highest dilution that achieved at least 50% neutralisation of the virus growth. All samples were tested in triplicate.

HAI and neutralisation assays were done with the homologous A/Vietnam/1194/2004 NIBRG-14 recombinant vaccine strain, but to assess cross-reactive immunogenicity, serum samples were also tested with a heterologous A/Indonesia/5/2005 prototype vaccine strain generated by reverse genetics that was made available by the Centers for Disease Control and Prevention (CDC, Atlanta, USA) on recommendations by WHO.[25]

## Statistical analysis

Safety and immunogenicity were prospectively identified as co-primary objectives. The sample size estimation was based on HAI geometric mean titres (GMTs). By means of a conservative approach, the alpha error was corrected to allow up to six independent comparisons between vaccine groups to assess the effect of adjuvantation and haemagglutinin dose. At least 45 assessable participants per group (total 360) provided 96% power to detect a three-fold increase in GMT between two vaccine groups, assuming the common SD was 0·5 (in log units) and by use of Student's *t* test with a 0·008 two-sided significance level. Our target was to enrol 400 participants to reach at least 360 assessable individuals.

We described the occurrence of solicited and unsolicited adverse events and any serious adverse events. We summarised these data (point estimates and 95% CI) in the total vaccinated cohort in terms of the incidence, intensity, and relation to vaccination of solicited and

| | Total (n=400) | 3·8 µg (n=50) | 3·8 µg+ adjuvant (n=51) | 7·5 µg (n=50) | 7·5 µg+ adjuvant (n=50) | 15 µg (n=50) | 15 µg+ adjuvant (n=50) | 30 µg (n=50) | 30 µg+ adjuvant (n=49) |
|---|---|---|---|---|---|---|---|---|---|
| **Age (years)** | | | | | | | | | |
| Mean (SD) | 34·3 (12·8) | 33·7 (11·8) | 35·3 (13·3) | 33·0 (11·9) | 34·4 (13·8) | 35·1 (12·8) | 33·9 (14·0) | 35·3 (13·1) | 33·6 (11·7) |
| Median (range) | 30·5 (18–60) | 31·0 (18–57) | 30·0 (19–60) | 30·5 (18–58) | 28·5 (18–58) | 30·5 (19–58) | 29·5 (18–60) | 31·0 (18–59) | 32·0 (18–58) |
| **Sex** | | | | | | | | | |
| Female | 217 (54%) | 27 (54%) | 36 (71%) | 25 (50%) | 22 (44%) | 25 (50%) | 30 (60%) | 27 (54%) | 25 (51%) |
| **Ethnic origin** | | | | | | | | | |
| White | 398 (100%) | 50 (100%) | 51 (100%) | 48 (96%) | 50 (100%) | 50 (100%) | 50 (100%) | 50 (100%) | 49 (100%) |
| African heritage | 1 (<1%) | 0 | 0 | 1 (2%) | 0 | 0 | 0 | 0 | 0 |
| Asian heritage | 1 (<1%) | 0 | 0 | 1 (2%) | 0 | 0 | 0 | 0 | 0 |

Data are number (%) unless otherwise indicated.

*Table 1:* **Baseline characteristics by vaccine group**

| | 3·8 µg (n=50) | 3·8 µg+adjuvant (n=51) | 7·5 µg (n=50) | 7·5 µg+adjuvant (n=50) | 15 µg (n=50) | 15 µg+adjuvant (n=50) | 30 µg (n=50) | 30 µg+adjuvant (n=49) |
|---|---|---|---|---|---|---|---|---|
| **Local (injection site) symptoms** | | | | | | | | |
| Pain | 19 (38%; 24·7–52·8) | 46 (90%; 78·6–96·7) | 21 (42%; 28·2–56·8) | 47 (94%; 83·5–98·7) | 21 (42%; 28·2–56·8) | 48 (96%; 86·3–99·5) | 34 (68%; 53·3–80·5) | 46 (94%; 83·1–98·7) |
| Redness | 9 (18%; 8·6–31·4) | 9 (18%; 8·4–30·9) | 5 (10%; 3·3–21·8) | 13 (26%; 14·6–40·3) | 5 (10%; 3·3–21·8) | 10 (20%; 10·0–33·7) | 5 (10%; 3·3–21·8) | 9 (18%; 8·8–32·0) |
| Swelling | 4 (8% 2·2–19·2) | 10 (20%; 9·8–33·1) | 1 (2%; 0·1–10·6) | 11 (22%; 11·5–36·0) | 3 (6%; 1·3–16·5) | 9 (18%; 8·6–31·4) | 4 (8%; 2·2–19·2) | 7 (14%; 5·9–27·2) |
| Induration | 5 (10%; 3·3–21·8) | 14 (28%; 15·9–41·7) | 2 (4%; 0·5–13·7) | 15 (30%; 17·9–44·6) | 1 (2%; 0·1–10·6) | 12 (24%; 13·1–38·2) | 3 (6%; 1·3–16·5) | 16 (33%; 19·9–47·5) |
| Ecchymosis | 4 (8%; 2·2–19·2) | 8 (16%; 7·0–28·6) | 0 (0%; 0·0–7·1) | 5 (10%; 3·3–21·8) | 4 (8%; 2·2–19·2) | 5 (10%; 3·3–21·8) | 2 (4%; 0·5–13·7) | 2 (4%; 0·5–14·0) |
| **General symptoms** | | | | | | | | |
| Arthralgia | 5 (10%; 3·3–21·8) | 14 (28%; 15·9–41·7) | 1 (2%; 0·1–10·6) | 11 (22%; 11·5–36·0) | 4 (8%; 2·2–19·2) | 13 (26%; 14·6–40·3) | 3 (6%; 1·3–16·5) | 6 (12%; 4·6–24·8) |
| Fatigue | 14 (28%; 16·2–42·5) | 23 (45%; 31·1–59·7) | 19 (38%; 24·7–52·8) | 28 (56%; 41·3–70·0) | 19 (38%; 24·7–52·8) | 30 (60%; 45·2–73·6) | 17 (34%; 21·2–48·8) | 27 (55%; 40·2–69·3) |
| Fever* | 0 (0%; 0·0–7·1) | 2 (4%; 0·5–13·5) | 2 (4%; 0·5–13·7) | 4 (8%; 2·2–19·2) | 1 (2%; 0·1–10·6) | 6 (12%; 4·5–24·3) | 1 (2%; 0·1–10·6) | 3 (6%; 1·3–16·9) |
| Headache | 18 (36%; 22·9–50·8) | 27 (53%; 38·5–67·1) | 16 (32%; 19·5–46·7) | 23 (46%; 31·8–60·7) | 19 (38%; 24·7–52·8) | 29 (58%; 43·2–71·8) | 19 (38%; 24·7–52·8) | 22 (45%; 30·7–59·8) |
| Muscle aches | 8 (16%; 7·2–29·1) | 20 (39%; 25·8–53·9) | 6 (12%; 4·5–24·3) | 20 (40%; 26·4–54·8) | 6 (12%; 4·5–24·3) | 24 (48%; 33·7–62·6) | 11 (22%; 11·5–36·0) | 23 (47%; 32·5–61·7) |
| Shivering | 6 (12%; 4·5–24·3) | 10 (20%; 9·8–33·1) | 5 (10%; 3·3–21·8) | 11 (22%; 11·5–36·0) | 2 (4%; 0·5–13·7) | 14 (28%; 16·2–42·5) | 4 (8%; 2·2–19·2) | 8 (16%; 7·3–29·7) |
| Sweating | 5 (10%; 3·3–21·8) | 9 (18%; 8·4–30·9) | 8 (16%; 7·2–29·1) | 16 (32%; 19·5–46·7) | 6 (12%; 4·5–24·3) | 13 (26%; 14·6–40·3) | 12 (24%; 13·1–38·2) | 11 (22%; 11·8–36·6) |

Data are number (%; 95% CI) *Fever defined as axillary temperature ≥37·5°C.

*Table 2:* **Participants with solicited local (injection site) and general symptoms within 7 days after one or both vaccinations by vaccine group**

unsolicited symptoms. We used the two-sided Fisher's exact test to compare groups where appropriate.

We summarised immunogenicity data in the per-protocol population. In addition to GMT (with 95% CI), other endpoints were based on HAI licensure criteria set out by the CHMP[11,26] and proposed in a draft guideline by the US Food and Drug Administration.[27] These endpoints (with 95% CI) included the mean geometric increase (defined as the ratio of GMTs after and before vaccination);[26] the percentage of participants with reciprocal titre of 40 or more after vaccination (deemed to be the seroprotective threshold[26,27] for seasonal vaccines); and the proportion of participants who seroconverted (defined as the percentage of people vaccinated who have a reciprocal titre of less than 10 before vaccination and a titre more than 40 after vaccination), or showed a significant increase in antibody titre (defined as the percentage of people vaccinated who had a reciprocal titre more than 10 before vaccination and at least a four-fold increase in titre after vaccination).

An ANOVA model was used to test the effect of haemagglutinin dose and adjuvantation. If there was non-significant interaction between these two factors, we used a factorial design approach to assess their effect. For the comparison between groups, 95% CI for HAI GMT ratio between adjuvanted-vaccine groups was calculated by use of a one-way ANOVA model on the $\log_{10}$-transformed titres. The ANOVA model included the vaccine-group effect. The GMT ratio was derived from the contrast of the vaccine-group effect. For neutralisation, the endpoints were GMT and seroconversion rate at every time point (with 95% CI).

This trial is registered with the ClinicalTrials.gov, number NCT00309634.

## Role of the funding source

GSK Biologicals was involved in the study design, data collection, data analysis, data interpretation, and writing of the report. The corresponding author had full access

to all the data in the study, participated in the interpretation of data and preparation of the report, and had final responsibility for the decision to submit for publication.

## Results

Between March 27 and June 15, 2006, 400 healthy adults were enrolled, received the two planned vaccinations, and completed the study (figure 1). All participants were included in the safety analysis and six were excluded from the immunogenicity analysis. The baseline characteristics were much the same in all study groups except that there were more women in the group assigned to the adjuvanted 3·8 µg antigen dose (table 1).

All eight vaccine formulations were well tolerated, and no immediate allergic reactions or other serious adverse events were reported during the trial. Table 2 shows the rates of individual solicited symptoms reported during the 7 days after vaccination. Pain at the injection site was the most common local symptom in all groups but it was reported significantly more frequently by participants who received the adjuvanted vaccine than by those who received the non-adjuvanted vaccine ($p<0·0001$ for 3·8 µg, 7·5 µg, and 15 µg formulations, and $p=0·002$ for 30 µg formulations). Pain at the injection site of grade 3 intensity was rare (reported by a total of four participants from the groups that received the 15 µg and 30 µg adjuvanted formulations). Other injection-site symptoms were less common. Generally, the rates tended to be higher in people who received the adjuvanted vaccine than in those who received non-adjuvanted formulations. Most symptoms were mild to moderate in intensity (data not shown). Injection-site redness, swelling, or induration of more than 50 mm was reported more frequently in the 7·5 µg and 15 µg adjuvanted groups than in all other groups. Most of these events resolved or decreased in intensity within 48 h.

The general symptoms most commonly reported were fatigue and headache, which tended to be more frequent in the adjuvanted-vaccine groups than in the non-adjuvanted-vaccine groups (table 2). The same pattern was recorded for arthralgia, fever, muscle aches, shivering, and sweating. Most reported general solicited symptoms were mild or moderate in intensity (data not shown). No participants had a temperature above 39°C, and only one had a temperature above 38°C for longer than 24 h. For all other symptoms, either no participants or only one per group reported events preventing normal activity that were thought to be related to vaccination, with the exception of fatigue in the adjuvanted 15 µg group (three participants) and shivering in the adjuvanted 3·8 µg group (two participants).

Other descriptive observations on solicited symptoms were that the frequencies seemed to be independent of

| | CHMP acceptance criteria* | FDA acceptance criteria† | Haemagglutinin dose and type of H5N1 vaccine (non-adjuvanted or adjuvanted) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 3·8 µg | 3·8 µg+ adjuvant | 7·5 µg | 7·5 µg+ adjuvant | 15 µg | 15 µg+ adjuvant | 30 µg | 30 µg+ adjuvant |
| **Antibody response variables (95% CI) at 21 days after first vaccine dose (day 21)** | | | | | | | | | | |
| GMT | No standard | No standard | 5·1 (4·9–5·4) | 12·9 (8·9–18·7) | 6·8 (5·4–8·7) | 24·6 (15·8–38·4) | 10·4 (6·9–15·6) | 24·7 (14·8–41·4) | 14·1 (8·9–22·6) | 36·7 (22·7–59·3) |
| Mean geometric increase (ratio day 21 GMT/day 0 GMT) | >2·5 | No standard | 1·0 (1·0–1·1) | 2·4 (1·7–3·5) | 1·4 (1·1–1·7) | 4·6 (3·0–7·0) | 1·9 (1·3–2·8) | 4·9 (2·9–8·1) | 2·7 (1·7–4·3) | 7·1 (4·3–11·7) |
| Seroconversion rate or significant increase in titre‡ | >40% | LL of 95% CI >40% | 0 (0%; 0·0–7·1) | 12 (24%; 13·1–38·2) | 4 (8%; 2·3–19·6) | 25 (50%; 35·5–64·5) | 10 (20%; 10·2–34·3) | 24 (49%; 34·4–63·7) | 13 (26%; 14·9–41·1) | 28 (58%; 43·2–72·4) |
| Seroprotection rate (titre ≥40) | >70% | LL of 95% CI >70% | 0 (0%; 0·0–7·1) | 13 (26%; 14·6–40·3) | 4 (8%; 2·3–19·6) | 25 (50%; 35·5–64·5) | 10 (20%; 10·2–34·3) | 24 (49%; 34·4–63·7) | 14 (29%; 16·6–43·3) | 28 (58%; 43·2–72·4) |
| **Antibody response variables with (95% CI) at 21 days after second vaccine dose (day 42)** | | | | | | | | | | |
| GMT | No standard | No standard | 6·2 (5·3–7·4) | 149·3 (93·2–239·1) | 8·5 (6·3–11·5) | 205·3 (135·1–312·0) | 14·7 (9·6–22·4) | 306·7 (218·4–430·8) | 20·0 (12·5–32·1) | 187·5 (116·2–302·7) |
| Ratio day 42 GMT/day 21 GMT | No standard | No standard | 1·2 (1·1–1·4) | 11·6 (6·8–19·6) | 1·2 (1·2–1·4) | 8·3 (5·3–13·2) | 1·4 (1·1–1·8) | 12·4 (7·5–20·6) | 1·4 (1·1–1·8) | 5·1 (3·3–7·9) |
| Mean geometric increase (ratio day 42 GMT/day 0 GMT) | >2·5 | No standard | 1·2 (1·1–1·5) | 27·9 (17·2–45·2) | 1·7 (1·3–2·3) | 38·1 (24·8–58·4) | 2·8 (1·9–4·1) | 60·5 (42·8–85·5) | 3·9 (2·4–6·2) | 36·4 (22·7–58·5) |
| Seroconversion rate or significant increase in titre‡ | >40% | LL of 95% CI >40% | 2 (4%; 0·5–13·7) | 41 (82%; 68·6–91·4) | 8 (16%; 7·3–29·7) | 45 (90%; 78·2–96·7) | 17 (35%; 21·7–49·6) | 47 (96%; 86·0–99·5) | 20 (41%; 27·0–55·8) | 41 (85%; 72·2–93·9) |
| Seroprotection rate (titre ≥40) | >70% | LL of 95% CI >70% | 2 (4%; 0·5–13·7) | 42 (84%; 70·9–92·8) | 8 (16%; 7·3–29·7) | 45 (90%; 78·2–96·7) | 17 (35%; 21·7–49·6) | 47 (96%; 86·0–99·5) | 21 (43%; 28·8–57·8) | 41 (85%; 72·2–93·9) |

GMT=geometric mean titre. LL=lower limit.*European Union Committee for Medicinal Products for Human Use (CHMP) criteria for haemagglutination-inhibition antibody response for people aged 18–60 years. †US Food and Drug Administration (FDA) proposed criteria for haemagglutination-inhibition antibody response in adults younger than 65 years. ‡Seroconversion rate for haemagglutination-inhibition antibody response is defined as the percentage of vaccinees who have a titre before vaccination of less than 1:10 and a titre after vaccination of 1:40 or more, or a significant increase in antibody titre is defined as the percentage of vaccinees who have a titre before vaccination of 1:10 or more and at least a four-fold increase in titre after vaccination.

*Table 3:* **Haemagglutination-inhibition antibody response to the homologous recombinant A/Vietnam/1194/2004 NIBRG-14 vaccine strain after the first and second vaccine dose**

antigen content (with the exception of injection-site pain in the non-adjuvanted groups which was higher in the 30 μg dose group) and that the frequencies and intensities after the second dose were similar to those observed after the first dose (data not shown).

The number of participants reporting at least one unsolicited symptom was similar in adjuvanted and non-adjuvanted vaccine groups (3·8 μg 28 [55%] *vs* 28 [56%], 7·5 μg 26 [52%] *vs* 28 [56%], 15 μg 36 [72%] *vs* 26 [52%], 30 μg 22 [45%] *vs* 23 [46%]). Unsolicited symptoms regarded as related to vaccination were reported more frequently in the adjuvanted than in the non-adjuvanted vaccine groups (3·8 μg 15 [29%] *vs* 5 [10%], 7·5 μg 12 [24%] *vs* 4 [8%], 15 μg 19 [38%] *vs* 6 [12%], 30 μg 7 [14%] *vs* 6 [12%]). However, only six of these symptoms were of an intensity that prevented normal activities, and all fully resolved. Swollen or enlarged axillary or supraclavicular lymph nodes were reported by seven participants in the adjuvanted groups, and in all but one the swelling was thought to be related to vaccination. All but two cases were recorded as easily tolerated (duration 4–13 days). The others were reported as interfering with normal activity (duration 2–4 days). Only one participant (who received a non-adjuvanted formulation) reported an allergy (allergic dermatitis), but it was not thought to be related to vaccination.

Before vaccination, most participants did not have detectable HAI antibodies against H5N1 (seven had HAI titres of 10 or more, of whom only three had titres above 40). GMTs before vaccination were much the same across the eight study groups (data not shown). Table 3 and figure 2 show HAI responses after the first and second vaccinations. A haemagglutinin dose-response relation was seen for both the adjuvanted and non-adjuvanted groups after the first dose ($p<0·0001$). After the second dose, a dose-response effect was noted for the non-adjuvanted groups ($p<0·0001$), whereas no dose effect was seen between the adjuvanted groups.

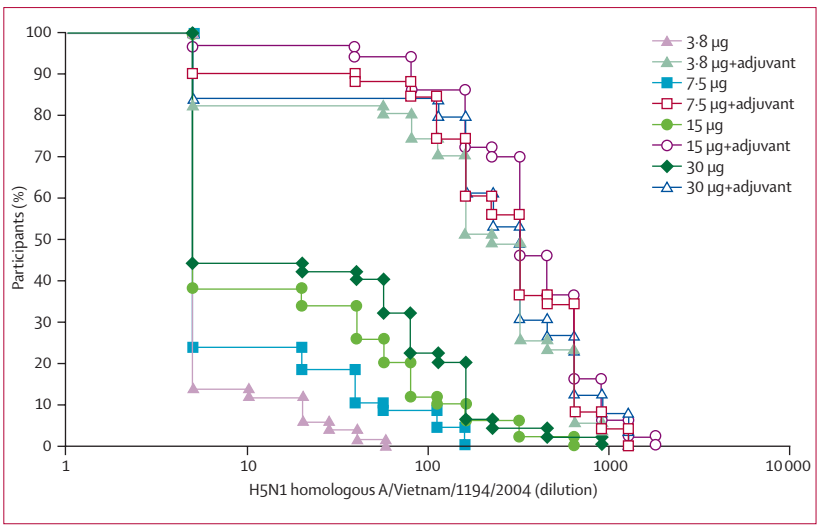


***Figure 2:*** **Reverse cumulative distribution curve for haemagglutination-inhibition antibody titres to the homologous recombinant A/Vietnam/1194/2004 NIBRG-14 vaccine strain after the second vaccine dose (day 42)**
GMT=geometric mean titre.

The GMTs indicate that a clear adjuvant effect was observed after each of the two vaccine doses, which was much more apparent after the second dose (table 3). The adjusted GMT ratio between groups for the adjuvantation effect showed that it improved the humoral response in terms of HAI titres after both vaccinations ($p<0·0001$). After each vaccine dose, both seroconversion and seroprotection rates were higher in the adjuvanted groups than in the corresponding non-adjuvanted groups (table 3). After the first vaccine dose, the CHMP criterion for seroconversion rate (>40%) was met by adjuvanted formulations containing antigen doses that were 7·5 μg or more but by none of the non-adjuvanted formulations (table 3). After the second vaccine dose, the adjuvanted formulations at all dose levels complied with both CHMP and FDA criteria for

| | 3·8 μg | 3·8 μg+ adjuvant | 7·5 μg | 7·5 μg+ adjuvant | 15 μg | 15 μg+ adjuvant | 30 μg | 30 μg+ adjuvant |
|---|---|---|---|---|---|---|---|---|
| **Antibody response variables with (95% CI) at 21 days after first vaccine dose (day 21)** | | | | | | | | |
| Number analysed | 50 | 50 | 49 | 49 | 48 | 49 | 49 | 47 |
| GMT | 35·5 (27·8–45·4) | 117·9 (93·7–148·3) | 40·3 (31·2–52·1) | 134·6 (101·3–178·7) | 66·9 (47·9–93·4) | 181·3 (144·6–227·3) | 80·1 (61·0–105·3) | 146·6 (113·3–189·3) |
| Seroconversion (four-fold increase in titre) | 7 (14%; 5·8–26·7) | 33 (66%; 51·2–78·8) | 11 (22%; 11·8–36·6) | 31 (63%; 48·3–76·6) | 23 (48%; 33·3–62·8) | 41 (84%; 70·3–92·7) | 28 (57%; 42·2–71·2) | 39 (83%; 69·2–92·4) |
| **Antibody response variables with (95% CI) at 21 days after second vaccine dose (day 42)** | | | | | | | | |
| Number analysed | 50 | 49 | 49 | 50 | 49 | 49 | 48 | 47 |
| GMT | 40·7 (32·4–51·0) | 314·7 (243·1–407·3) | 53·4 (41·6–68·6) | 343·0 (260·5–451·5) | 80·1 (60·1–107·0) | 400·1 (319·3–501·4) | 113·6 (85·5–150·9) | 258·2 (205·5–324·5) |
| Seroconversion (four-fold increase in titre) | 11 (22%; 11·5–36·0) | 42 (86%; 72·8–94·1) | 18 (37%; 23·4–51·7) | 43 (86%; 73·3–94·2) | 26 (53%; 38·3–67·5) | 42 (86%; 72·8–94·1) | 31 (65%; 49·5–77·8) | 46 (98%; 88·7–99·9) |

GMT=geometric mean titre.

***Table 4:*** **Neutralising antibody response to the homologous recombinant A/Vietnam/1194/2004 NIBRG-14 vaccine strain after the first and second vaccine dose by vaccine group**

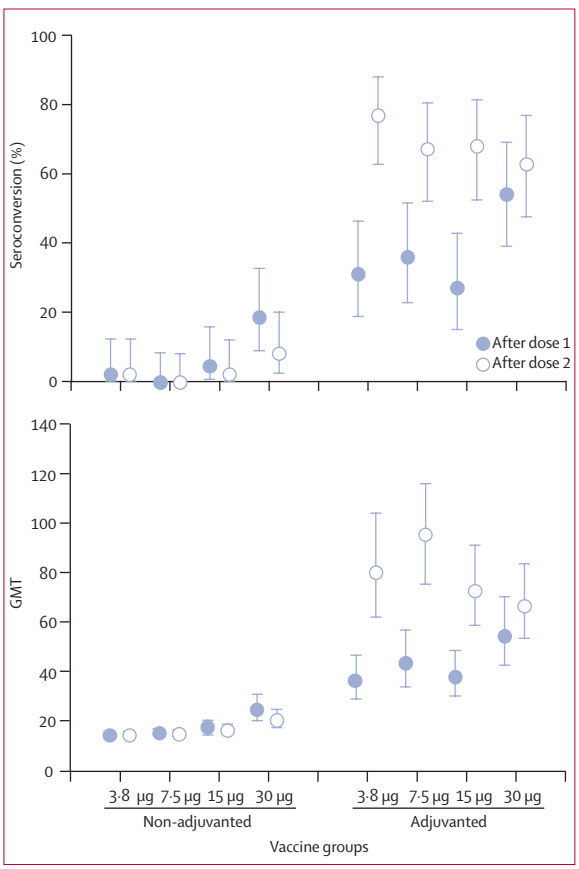


***Figure 3:*** **Seroconversion (upper) and GMT (lower) neutralising antibody response to the heterologous recombinant A/Indonesia/5/2005 strain after the first and second vaccine dose**
GMT=geometric mean titre. Error bars show 95% CIs.

seroconversion and seroprotection rates, whereas from the non-adjuvanted groups only the 30 µg formulation met the CHMP criterion for seroconversion rate (table 3). The mean geometric increase after the first dose in the adjuvanted groups were sufficient to meet the CHMP acceptance criterion of more than 2·5 for antigen doses from 7·5 µg, whereas only the 30 µg dose achieved this criterion in the non-adjuvanted groups (table 3). After the second dose, the mean geometric increase in the adjuvanted groups greatly exceeded the CHMP criterion at all antigen doses, but neither of the lower doses achieved this criterion in the non-adjuvanted groups (table 3).

Adjuvantation substantially improved neutralising antibody responses in vitro in the adjuvanted groups compared with the non-adjuvanted groups, with differences of five to eight times in GMTs recorded after the second dose for the 3·8 µg, 7·5 µg, and 15 µg antigen formulations (table 4). An antigen dose-response relation was seen for the non-adjuvanted formulations ($p<0·0001$) but this relation was not evident for the adjuvanted formulations ($p=0·095$). The adjuvant effect was also evident in the neutralising seroconversion rates and was most pronounced at the lowest antigen levels after the second dose (table 4).

To assess the vaccine's ability to induce cross-reactivity against drifted H5N1 strains, we investigated humoral immunity against a strain derived by reverse genetics from a drifted H5N1 isolate A/Indonesia/5/2005 (clade 2). After the second dose, seroprotective HAI titres against this heterologous strain were induced in 20–32% of participants in the adjuvanted groups but in none of those in the non-adjuvanted groups (data not shown). However, in the more sensitive neutralisation assay, four-fold seroconversion responses were recorded against the Indonesia strain (figure 3), which were much higher than were the HAI responses. After the first dose, 12–25 (27–54%) of participants in the adjuvanted groups had seroconverted. This response increased to between 31 (67%) and 37 (77%) after the second dose, whereas in the non-adjuvanted groups, the neutralising seroconversion rate remained below 9%. A convincing adjuvantation effect on heterologous neutralising responses was further shown by the increased GMTs after the second dose, with differences of up five to six times more than in the corresponding non-adjuvanted groups for the antigen doses of 3·8 µg and 7·5 µg (figure 3). Again, no antigen dose-response relation was evident for the adjuvanted formulations ($p=0·76$).

## Discussion

We have shown that use of the oil-in-water based emulsion adjuvant system substantially reduces the amount of antigen needed to elicit a satisfactory immune response to a recombinant H5N1 influenza A/Vietnam/1194/2004 (clade 1) split-virion vaccine and induces cross-clade neutralising immunity against a strain derived by reverse genetics from the drifted A/Indonesia/5/2005 strain (clade 2). These findings are important since the identification of a candidate H5N1 pandemic influenza vaccine that can be commercially manufactured at large scale, is immunogenic at low antigen doses, and which confers cross-clade immunity against drifted H5N1 strains is an important global health objective.

Both the non-adjuvanted and adjuvanted vaccine formulations had a good safety profile across a wide age range of healthy adults. No serious adverse events were reported, and all participants completed the two-dose regimen. There was no evidence of an effect of H5 antigen dose on reactogenicity except for an increase in injection-site pain for the highest non-adjuvanted dose. Treanor and colleagues[15] also reported that after vaccination with non-adjuvanted H5N1 vaccine, the frequencies of pain and tenderness at the injection site increased in a dose-dependent way. Although the adjuvant regimen led to more frequently reported solicited injection-site and general adverse events than did the non-adjuvant regimen, most of these events were mild to moderate in intensity and resolved within 48 h.

Bresson and colleagues[17] showed that the addition of aluminium adjuvant to H5N1 vaccine led to an increased rate of local reactions but the general safety profile was not adversely affected. The frequencies of solicited reactions reported for the aluminium-adjuvanted vaccine were within the same range as those recorded for the adjuvanted vaccine in this study with the exception of pain at the injection site, myalgia, and shivering, which tended to be higher in our study than in that of Bresson and colleagues.[17] Another adjuvant that has been used to increase the immunogenicity of influenza antigens is MF59.[18,28] In phase 1 studies with MF59-adjuvanted vaccine of H5N3 (a non-pathogenic variant of H5N1) and H9N2 (another avian virus that can infect people), the investigators noted an increase in injection-site pain that was associated with the adjuvant.[18,29] Extensive clinical experience with MF59-adjuvanted interpandemic influenza vaccine has shown that it induced more reactions than did conventional comparator influenza vaccines.[29] Since these reactions were mostly mild to moderate and transient in nature, this decrease in tolerability was regarded as clinically acceptable in an interpandemic context with yearly vaccination.[28] In view of the high risk of serious illness and mortality associated with pandemic influenza and the substantial benefit of an effective vaccine, the reactogenicity profile of the candidate adjuvanted H5N1 vaccine that we recorded, is clinically acceptable.

As we expected, most of our participants were immunologically naive for H5N1 before vaccination. Despite the very low probability of previous exposure to H5 virus, the low percentage of participants with detectable or seroprotective concentrations of HAI antibodies accords with other studies of H5N1 vaccination.[15,17,19] One of these studies showed that two doses of non-adjuvanted split-virion H5N1 vaccine containing up to 90 µg of H5 antigen were needed to elicit a satisfactory immune response,[15] whereas another reported that aluminium adjuvant only modestly improved the immunogenicity of H5 doses below 30 µg.[17] Our study confirmed that two 30 µg doses of non-adjuvanted split-virion vaccine were needed to induce a HAI response that met at least two of the three CHMP criteria,[11,26] but neither of the proposed FDA criteria were met.[27] A strong booster effect of the adjuvanted vaccine was seen after the second dose, and responses in all adjuvant groups met or exceeded all CHMP and FDA criteria. The immunological licensure criteria for yearly interpandemic vaccines are based on an HAI assay that uses avian erythrocytes and assumes some degree of pre-existing immunity. Thus these same criteria, if applied to the assessment of pandemic vaccines in a largely naive population,[11] might not be applicable. In the absence of other criteria, however, we have to assume that an HAI titre of 40 or more, which was achieved by more than four-fifths of the recipients of the lowest dose of adjuvanted H5N1 vaccine, would probably predict clinical benefit.

The CHMP also recommends that neutralising antibodies should be measured for pandemic influenza vaccines.[11] Although no protective neutralising correlate has been established, the conventional four-fold increase in titre between sampling points before and after vaccination seemed reasonable and was used to show seroconversion. Although the neutralising response pattern was similar to that observed for HAI, the seroconversion rates were higher, especially after the first dose. The haemagglutination-inhibition results suggest that antibodies are produced that specifically block the haemagglutinin receptor-binding site involved in attachment of the virus to the host cell. The neutralisation data, however, confirm the production of biologically functional antibodies that can inhibit the complex process of virus attachment, entry, and release from cells in tissue culture.[21,30]

Most of the H5N1 strains in circulation in the past 3 years can be separated into two distinct phylogenetic clades on the basis of their haemagglutinin sequences. The A/Vietnam/1194/2004 vaccine strain belongs to clade 1, whereas most recent isolates belong to clade 2.[25,31] A/Indonesia/5/2005 is one example of a clade 2 virus for which a prototype reassortant strain engineered by reverse genetics has been made available by CDC, and this strain was used to assess cross-reactivity in our study.[25] Our data show that the adjuvanted H5N1 vaccine can induce a cross-reactive HAI and neutralising-antibody response to A/Indonesia/5/2005. The cross-reactivity was stronger when assessed by neutralising assay than by haemagglutination-inhibition assay, which contrasts with the responses to the A/Vietnam/1194/2004 NIBRG-14 vaccine strain that were similar by both assays after two doses. This finding might be related to the high antigenic variation at the H5 haemagglutinin site involved in viral attachment,[32] which is specifically measured by the HAI assay. This effect will be evaluated in ferret heterologous challenge studies which will measure the effect of the vaccine on virus replication in vivo. As suggested by other investigators,[18,21] the neutralisation assay, which was used to assess serological responses during the 1997 outbreak,[8,23] could be an important means by which responses to different H5 viruses could be assessed.

Whereas an antigen-dose effect was observed on the HAI response for the non-adjuvanted formulations after the second vaccination, no such effect was recorded for the adjuvanted formulations. There was also no antigen-dose response for the adjuvanted formulations for the neutralising response (against both vaccine and A/Indonesia/5/2005 strains). These findings suggest that the stimulatory effect of the adjuvant on the immune system compensates for the limiting effect of antigen concentration, thereby reducing the dose needed to give the maximum response.

Research on the mechanisms underlying the effect of adjuvants is in progress. On the basis of published data and other preliminary investigations (unpublished),

different mechanisms of actions could account for the immunostimulatory properties of the adjuvant used in this study. Indeed, in addition to their vehicle properties, emulsions that are oil-in-water based have been shown to induce local inflammation and attraction of immunocompetent cells at injection sites.[33,34] Furthermore, the plant-derived liposoluble vitamin E (ie, DL-α-tocopherol) included in this novel adjuvant system increases immune functions such as vaccine-induced production of antibodies mediated through an increased production of cytokine, leading to greater proliferation of T cells,[35,36] and through a reduced production of prostaglandin $PGE_2$ (a T-cell suppressive factor).[37,38] In this context, new studies are clarifying the vaccine's ability to induce cell-mediated immunity, but this work needs further validation.

Although this and other studies[15,17] of pandemic vaccines have focused on conventional split-virion vaccines, a parallel approach has been the development of formalin-killed whole-virion vaccines manufactured by a process that was previously used extensively for influenza vaccine production.[19,21,39] Although the main advantage of this type of vaccine over split-virion vaccines is an increased antigen yield, it is generally associated with a higher degree of reactogenicity than are split-virion vaccines. Lin and colleagues[19] have shown that the use of an H5N1 whole-virion vaccine that is adjuvanted with aluminium could result in an antigen-sparing effect, with a 10 μg dose meeting all CHMP criteria.[19] This finding accords with studies of a similar aluminium-adjuvanted whole-virion H5N1 vaccine, which suggest that the minimum dose needed to meet all CHMP criteria is between 7·5 μg and 15 μg.[39] However, investigations have shown that the neutralising activity of the aluminium-adjuvanted whole-virion H5N1 vaccine against the vaccine strain is lower than that of the adjuvanted split-virion H5N1 vaccine (unpublished data). Studies are in progress to establish whether this is confirmed for cross-reactive neutralising activity against the reassortant A/Indonesia/5/2005 strain.

The ability of the 3·8 μg dose adjuvanted split-virion vaccine to induce cross-immunity against the clade 2 Indonesia strain in more than three-quarters (seroconversion rate) of individuals with a GMT that is six times higher than the non-adjuvanted formulation, represents significant antigen sparing that could increase the number of recipients of the pandemic influenza vaccine. The cross-clade neutralising antibody responses recorded imply that such a vaccine could be deployed before pandemic outbreak, which is an important mitigation strategy proposed for pandemic influenza.[40,41] However, further analyses are warranted to confirm whether it could also confer high degrees of cross-protection against other pandemic drift strains. The whole-virion approach should nevertheless be maintained as an important back-up strategy for the large-scale production of a matched pandemic vaccine once a specific pandemic strain has been identified.

**Contributors**

All authors participated in the design, implementation, analysis, and interpretation of the study. IL-R, AB, MD, J-MD, and GL-R were involved in all phases of the study. IL-R led the clinical team at the Centre for Vaccinology, assisted by TV and EH. AB and J-MD led the clinical team at GSK Biologicals. MD did the data analysis. GL-R was the principal investigator. FC managed the team responsible for handling and processing of the blood samples.

**Conflict of interest statement**

AB, JMD, and MD are employees of GSK Biologicals. JMD owns stock from GSK. None of the other authors in this report declares a conflict of interest.

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